GHB and GBL from Biological Fluids by Headspace GC/MS(EI)

1 Introduction

Gamma-hydroxybutyrate (GHB) and its precursors, gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD), have become popular recreational substances in the United States. This is primarily due to their sedative and euphoric properties, but they are also abused as steroid alternatives by some bodybuilders. Furthermore, these chemicals have been implicated in a number of cases of drug-facilitated sexual assault.

Both GBL and 1,4-BD are rapidly metabolized to GHB *in vivo*, thus the pharmacological effects of both mimic those of GHB. The conversion is so rapid that in most cases it is not necessary to analyze for GBL and 1,4-BD. However, in overdose cases a GHB finding may be explored for the possibility of GBL or 1,4-BD involvement. This procedure allows for the analysis of a specimen for GHB and GBL.

There are a number of analytical complications surrounding GHB. One such complication is its existence as an endogenous product in humans. In postmortem specimens, the GHB levels in blood may be elevated to such a degree that apparent endogenous levels of GHB may overlap those reported to be lethal. An added complication is the pH-driven interconversion between GHB and GBL. Thus, caution should be employed in the interpretation of GHB findings.

2 Scope

This procedure allows for the screening and confirmation of GHB in urine specimens and aqueous samples. When appropriate controls are simultaneously analyzed, it can be used to screen blood samples for elevated levels of GHB. It also provides a method for the differentiation of GHB from GBL.

3 Principle

For the initial screen, d_6-GHB is added to the specimen as the internal standard. Since 10 µg/mL is the administrative cutoff for urine specimens, a 10 µg/mL GHB Positive Control is also extracted. The samples are treated with concentrated sulfuric acid and heat (for conversion of GHB to GBL). Methylene chloride is used to extract the GBL from the biological matrix. The organic extracts are concentrated and transferred to headspace autosampler vials. The vials are heated and the headspace is analyzed by gas chromatography/mass spectrometry (GC/MS). This method measures the total amount of GHB and GBL in a specimen. For urine specimens, if the ratio of the GHB peak area/internal standard peak area in the questioned sample approaches the ratio of the GHB peak area/internal standard peak area in the 10 µg/mL Positive Control, the sample is

presumed positive. For aqueous specimens, any amount of GHB detected is considered positive, although care should be given to the interpretation of the findings. If GBL is suspected in a sample, it may be analyzed without acid hydrolysis and with α-methylene GBL as the internal standard. This procedure may be repeated to confirm positive samples, and serves as a framework for quantitative analysis with proper validation.

4 Specimens

This procedure can be performed on a biological fluid such as blood, serum, plasma, urine, or vitreous humor. 2.0 mL of specimen are used in order to perform the screen and the confirmation.

5 Equipment/Materials/Reagents

a. Gas Chromatograph / Mass Spectrometer operating in electron impact (EI) mode equipped with a headspace autosampler and a 30 m x 0.25 mm x 1.4 µm film DB-624 (or equivalent) column

b. Centrifuge

c. Vortex mixer

d. Heating block

e Evaporator with nitrogen

f. Balance

g. Adjustable pipetters (0.01 - 1 mL) with appropriate tips

h. 16 x 100 mm screw-top test tubes with caps

i. 10 mL conical-bottom screw-top centrifuge tubes with caps

j. 10 mL and 100 mL volumetric flasks

k. Routine laboratory supplies, including disposable glass pipets, autosampler vials with caps, spatulas, graduated cylinders, test tube racks, etc.

l. Concentrated Sulfuric Acid (Reagent Grade)

m. Methylene Chloride (HPLC Grade)

n. Methanol (HPLC Grade)

o. Deionized water

p. Rotater

q. 20-mL headspace vials with magnetic caps

6 Standards and Controls

a. GHB Na Stock Standard (1.0 mg/mL):
Purchased from Cerilliant Corporation or another approved supplier as the sodium salt. Storage and stability determined by manufacturer.

b. GHB Na Working Standard (0.1 mg/ml):
Prepare by adding 1.0 mL of the GHB Na Stock Standard to a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated in glass. Stable for at least one year.

c. GBL Stock Standard (1.0 mg/mL):
Purchased from Cerilliant Corporation or another approved supplier. Storage and stability determined by manufacturer.

d. d_6-GHB Na Internal Standard (d_6-Gammahydroxybutyrate Sodium Salt; 100 µg/mL OR 1 mg/mL):
Purchased from Cerilliant Corporation or another approved supplier. Storage and stability determined by manufacturer.

e. α Methylene-GBL:
Purchased from Sigma or another approved supplier. Storage and stability determined by manufacturer.

f. α Methylene-GBL Internal Standard (0.1 mg/mL):
To a 100-mL volumetric flask, add 10.0 mg of Alpha Methylene- Gammabutyrolactone. Bring volume to the mark with methanol. Store refrigerated in glass or plastic. Stable for at least 1 year.

g. Negative Control:
Since GHB is endogenous, the most appropriate Negative Control is a deionized water sample or synthetic urine (Surine, obtained from Dyna-Tek, Inc., Lenexa, KS). Stability and storage of Surine are determined by the manufacturer.

However, a true matrix matched Negative Control (urine or blood) is also analyzed.

Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Blood and urine will be stored refrigerated, frozen or obtained fresh. Stability determined by manufacturer.

A Negative Control will be analyzed with every assay.

h. Positive GHB Controls (5 and 10 µg/mL):
For urine screens, Positive Urine Controls at 5 and 10 µg/mL are analyzed. These are prepared fresh by adding 60 and 120 µL of the GHB Na Working Standard to 1 mL aliquots of Surine.

At least one Positive GHB Control will be analyzed with every GHB assay.

i. Positive GBL Control (10 µg/mL):
Prepared fresh by adding 10 µL of the GBL Stock Standard (1.0 mg/mL) to 1 mL of Surine.

A Positive Control (GBL) will be analyzed with every GBL assay.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the Examiner or Chemist performing the procedure.

a. Add 1 mL specimen or control to a properly labeled 16 x 100 mm screw-top test tube.

b. Add 10 µg d_6-GHB Na (10 µL of the 1.0 mg/mL d_6-GHB Na Internal Standard or 100 µL of the 100 µg/mL d_6-GHB Na Internal Standard) to each tube[1].

c. Add 150 µL of concentrated sulfuric acid to all tubes, cap, vortex, and place them in a heating block at approximately 70°C for 5 minutes. Cool to room temperature.

d. Add 4 mL of methylene chloride to each tube. Extract by rotation for 5 minutes; centrifuge for 5 minutes.

e. Remove aqueous (top) layer to waste. Transfer organic (bottom) layer to an appropriately labeled conical bottom tube.

[1] To screen for GBL, add 20 µL of the 0.1 mg/mL α-methylene-GBL Internal Standard instead of d_6-GHB, and skip to step d.

f. Concentrate organic layer to approximately 75 µL in the evaporator under nitrogen at approximately 35°C. Do not let the organic layer go to dryness.

g. Transfer concentrated organic to a 20-mL headspace vial and cap.

h. Analyze specimens by headspace GC/MS with the instrumental parameters listed later in this procedure.

9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Headspace Sampler Parameters

incubation temperature	100°C	syringe temperature	110°C
incubation time	15 min	sample fill volume	1.0 mL
agitator speed	300 RPM	sample fill rate	0.5 mL/sec
agitation timing	10 sec on 1 sec off	sample fill strokes	4
cycle time	20 min	sample injection speed	1.0 mL/sec
		syringe flush time	4.0 min

9.2 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature 1	50°C	type	DB-624	inlet temp.	150°C
hold 1	3 min	length	30 m	injection mode	split
ramp 1	20°C/min	internal diameter	0.25 mm	carrier gas	helium
temperature 2	150°C	film thickness	1.4 µm		
hold 2	7 min			carrier mode	constant flow
total run time	15 min			flow	0.87 mL/min
				split ratio	10:1

9.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	260°C
scan range	35 - 200 AMU	quadrupole temperature	150°C
multiplier offset	+200 V	solvent delay	5 min

10 Decision Criteria

10.1 GHB Screen: Determining if Further Analysis is Required

When screening a urine sample to determine if a toxicologically significant amount of GHB is present, calculate the ratio of the GHB peak area/internal standard peak area in all samples. (This may be done automatically using the software on the instrument, or manually.) If the area ratio in the questioned urine sample approaches the area ratio in the 10 µg/mL Positive Control, the sample is presumed positive. Blood samples are not routinely analyzed by this procedure, but a level estimated to be above 2 µg/mL should be investigated further.

10.2 Decision Criteria for Analytical Data

The following criteria are used as guides in determining the acceptability of the data produced in this assay. In general, compound identification should be based on comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control. In most cases, all of the below should be met in order to identify GHB or GBL within a biological specimen.

10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.2.1.1 Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from the injection of a reference standard, calibrator, or extracted Positive Control of GHB or GBL.

10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank injected just prior to that sample.

10.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of the appropriate reference

standard, calibrator, or extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

11 Calculations

To calculate the ratio of the GHB peak area to the internal standard peak area, reconstructed ion chromatograms are traced for m/z 86 (GHB) and m/z 92 (d_6-GHB). The traces are integrated, resulting in an area for each peak. The area of the 86 peak divided by the area of the 92 peak is the peak area ratio used in Section 11.1 of the Decision Criteria Section of this procedure.

12 Measurement Uncertainty

Not applicable.

13 Limitations

a. Limit of Detection: Although levels of GHB less than 5 µg/mL can be analytically identified in urine samples, the limit of detection is administratively set at 5 µg/mL in urine samples.

b. Interferences: None known. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.

14 Precautionary Statement

Care should be taken in interpretation of GHB levels. GHB is a naturally occurring product in the body. Further, studies have shown that GHB is elevated in blood collection tubes containing citrate. Exercise care in reporting and interpreting low values of GHB.

When analyzing specimens from living persons, in most cases, amounts of GHB in blood below 2 µg/mL, and/or amounts of GHB in urine below 10 µg/mL should not be reported as positive.

GHB levels may be elevated in postmortem blood samples and/or unpreserved blood samples. Therefore, a positive GHB finding in a postmortem blood sample should always be confirmed in a second specimen such as urine or vitreous humor.

15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

16 References

Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed.; Chemical Toxicology Institute, Foster City, California, 1995.

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Stevens. *J For Sci*. 1999; *44*(1), 231-2.

Guidelines for Toxicological Quantitations (Tox 101)**;** FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Preparation of Chemical Reagents (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Division Safety Manual.

Rev. #	Issue Date	History
3	09/19/12	Removed reference to 1,4-BD SOP in Section 1 (SOP archived). Removed all references to quantitative analysis of GHB and GBL in Sections 2, 3, 9 (9.1 header designating screen and entire 9.2 removed), 12, 13, 14 and 17. Updated GHB and GBL stock standards in Sections 6 a, b and c to Cerilliant. Updated Control preparation in Sections 6 g and h to include two levels of GHB control and not to require volumetric flask since assay qualitative only now. In Section 9 b, added footnote for GBL screening. Updated decision criteria for the screen in 11.1. Updated chromatography decision criteria in Section 11.2.1. Added note about possibility of elevated GHB levels in unpreserved blood in Section 15.
4	07/09/14	In Section 6.f, added sources for Negative Control blood. Fixed typo in Section 10.2 (flow rate). On Appendix 1, added option for GBL analysis and a spot to record dry down temperature. Reformatted Appendix 2 to include all pertinent instrumental parameters.
5	04/20/15	Changed GBL internal standard to α-methylene-GBL since d_6-GBL is no longer commercially available. This caused updates to the following Sections: 3, 6.e., 6.f., 8.b.(footnote), and Appendix 1.In Section 5, removed reference to Tox 103 (reagent SOP). Removed Calibration Section. Renamed Measurement Uncertainty Section.

Approval

Redacted - Signatures on File

Appendix 1: Abbreviated version of the GHB Procedure for bench use.

Redacted - Form on File

Appendix 2: Abbreviated version of Instrumental Parameters for bench use.

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